A study of expression and function of the transcription factor *Skor2* in glutamatergic neurons of the developing brainstem

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Tiivistelmä – Referat – Abstract

The brainstem monoaminergic neuronal systems are involved in regulation of mood, reward system, memory processing etc. Any defects or damage in these cells lead to many neurological disorders. The brainstem inhibitory GABAergic and excitatory glutamatergic cells in turn control these neuromodulatory neurons. The glutamatergic neurons are found in the Laterodorsal tegmental nucleus (LDTg), Interpeduncular nucleus (IPN) as well as in the Ventral tegmental are (VTA). The LDTg in particular sends these glutamatergic projections to the VTA to regulate their Dopaminergic (DA) neurons. During embryonic development, the brainstem GABAergic and glutamatergic neurons, that regulate the monoaminergic systems, are produced in the ventral rhombomere 1. Their subtypes are known to express various transcription factors (TFs), such as *Nkx6-1, Vsx2* and *Skor1* marking the glutamatergic neuron precursors in the ventral rhombomere 1.

In this thesis project, I studied the expression of another TF, *Skor2* in the embryonic brainstem precursors. The basis of the experiment came from an embryonic brainstem single cell mRNA sequencing study performed earlier, where *Skor2* expression was observed in the cluster of neurons containing *Nkx6-1, Vsx2* and *Skor1* expressing cells. Based on this, I hypothesized that *Skor2* expression could be seen in glutamatergic precursors in the ventral rhombomere (rV2) domain as well as later in the LDTg nucleus derived from these precursors. To test this, I performed immunohistochemistry (IMHC) studies on a transgenic mouse line expressing *G*reen Fluorescent Protein (*GFP)* from the *Skor2* locus. In the second part of the thesis, I hypothesized that the *Skor2* positive cells need this TF for their differentiation. To study this aspect, I performed similar IMHC studies on homozygous Skor2GFP/GFP mice, where *Skor2* had been inactivated.

My study showed that *Skor2* positive cells expressed markers *Nkx6-1* and *Vsx2* and represented a specific subgroup of early embryonic post-mitotic precursors in the rV2 domain. Later in the brainstem, in contrast to my initial hypothesis, I did not observe *Skor2* expression in the LDTg glutamatergic region. Instead, I observed *Skor2* positive cells in a region more lateral to the Ventral and Dorsal tegmental nuclei of Gudden. In the homozygous *Skor2* mutants, I observed no changes in cell fate during embryonic development.

Based on my results, the TF *Skor2* is expresses in the glutamatergic precursors and neurons in the rhombomere 1, but form a part of a new cluster of cells away from the LDTg. These neurons have not been studied in detail. However, the Ventral and Dorsal tegmental nuclei of Gudden have been shown to regulate memory and navigation. It is possible that the Skor2 expressing neurons also participate in these functions. Identification of specific molecular markers, such as Skor2, for these neurons now allows their focused functional studies. *Skor2* and *Skor1* are related TFs belonging to *Ski* family of transcriptional repressors and are seen to be expressed together. Further investigations into the roles and functional redundancy of these two TFs can be performed using mice carrying mutations in both of these genes.

Avainsanat – Nyckelord – Keywords

Skor2, Laterodorsal tegmental nucleus, glutamatergic neurons, Ventral tegmental nucleus of Gudden, Dorsal tegmental nucleus of Gudden

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The project was undertaken in the Developmental Neurogenetics Lab, led by Juha Partanen

Abbreviations

5-HT – Serotonin

BMP – Bone morphogenetic protein

ChAT – Choline Acetyltransferase

DA – Dopamine

DTg – Dorsal Tegmental nucleus of Gudden

FUSSEL – Functional Smad suppressing element

GABA – gamma-Aminobutyric acid

GFP – Green Fluorescent Protein

IMHC – Immunohistochemistry

IPN – Interpeduncular nucleus

LDTg – Laterodorsal Tegmental nucleus

mDA – midbrain Dopaminergic neurons

LHb – Lateral Habenula

mz1 – Mantle zone 1

mz2 – Mantle zone 2

PC – Purkinje cell

PFA – Paraformaldehyde

r1 – rhombomere 1

RMTg – Rostromedial tegmental nucleus

RRF – Rostrorubral field

SN – Substantia Nigra

SNpc – Substantia Nigra pars compacta

SNpr – Substantia Nigra pars reticulata

TF – transcription factor

TH – Tyrosine Hydroxylase

VTA – Ventral Tegmental Area

VTg – Ventral Tegmental nucleus of Gudden

VZ – Ventricular Zone

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Results

Skor2 is expressed in post-mitotic rV2 precursors in the mz2 along with *Nkx6-1* at E11.5-E13.5

Skor2 has minimal co-expression with *Vsx2*, the latter predominantly marking the rV2 mz1 at E11.5 - E13.5

Skor2 is not expressed in the LDTg along with *Nkx6-1*, but shows expression in a region more ventral

Skor2 is predominantly expressed in *Vsx2* negative cells at E15.5 and E18.5

Anatomical characterization of *Skor2* expressing cells in the posterior brainstem: identification of *Skor2* positive neurons between the VTg and DTg

Skor2 deficient mice do not seem to have changes in the brainstem cell types

Discussion

Skor2 positive neurons label in rv2 domain of early embryonic tissues

Skor2 was not expressed in LDTg at later embryonic stages

Skor2 positive cells line up between DTg and VTg in later embryonic stages

Skor2 not required for differentiation of their neuronal subtype in the embryonic development

Possible future experiments

Acknowledgments

References

Introduction

The brainstem, the ventral part of the midbrain and anterior hindbrain, contains neurons regulating functions, such as mood, motivation and movement. These neurons include monoaminergic cell types as well as neurons, including inhibitory GABAergic and excitatory glutamatergic neurons, associated with them. Despite its important functions, the cell types of the brainstem, and their developmental origins and regulation, remain poorly understood. This thesis focuses on a subtype of brainstem glutamatergic neuronal precursors, marked by the expression of a transcription factor (TF) *Skor2.*

Dopaminergic and Serotonergic neurons in the anterior brainstem

Neuromodulatory neurons using dopamine (DA) or serotonin (5-HT) as their neurotransmitter are present in low numbers in the brain. Roughly, two-thirds of these DA neurons are found in the midbrain (Niederkofler, Vera et al, 2015). The DA neurons are part of three clusters: Ventral Tegmental Area (VTA), Substantia Nigra pars compacta (SNpc) and the retrorubral field (RRF). Alongside them, the 5-HT neurons are found in the hindbrain in the raphe nuclei. Midbrain Dopaminergic neuron (mDA) innervate into the striatum and cortex. Other target regions include hippocampus, lateral Habenula, amygdala and septum. The mDA project via three pathways: mesostriatal/nigrostriatal, mesolimbic and mesocortical pathways. The mesostriatal pathway involves SN mDA neurons projecting towards the dorsal striatum. This pathway is involved in voluntary motor control. The mesolimbic and mesocortical pathways both involve VTA mDA neurons and are responsible for modulating cognition, emotion and motivation. The difference between these pathways is the target destination of the VTA projections. In the mesolimbic pathway, the VTA DA neurons project to the nucleus accumbens in the ventral striatum. Mesocortical pathway involves VTA DA neurons that project to the cortical regions, as the name

implies. In contrast, the 5-HT pathways are broader and diffusely projected throughout the rostral and caudal brain. The projections are less defined (Aitken et. al, 1988; Molliver et.al, 1987) in comparison to the mDA neurons. These projections play a part in modulating respiration, thermoregulation, aggression and activity.

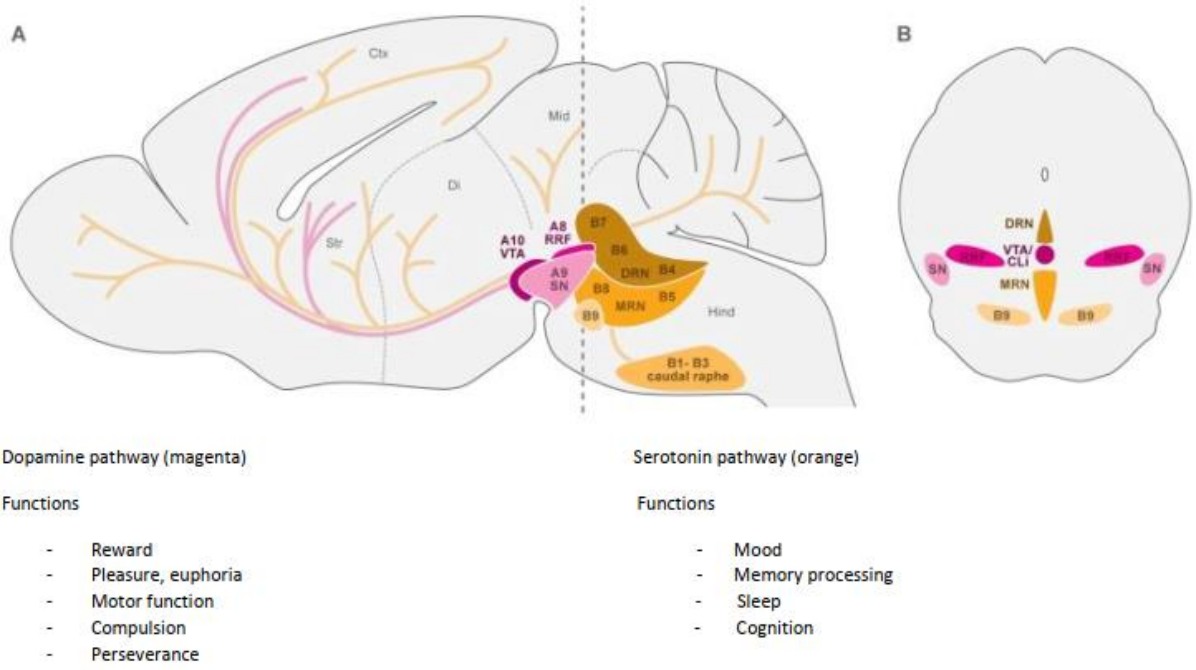


Figure 1: Anatomy of the midbrain DA and 5-HT neurons. The DA neurons effect their functions on the striatum, nucleus Accumbens (ventral striatum) and cortex. Their pathways are more defined in comparison to the 5-HT neuronal projections, which innervate most parts of the brain. (Modified from: Niederkofler, Vera et al, 2015)

GABAergic subtypes that play an inhibitory role in regulation of Dopaminergic and Serotonergic neuronal systems

While we understand the output nature of the DA and 5-HT nuclei, the input they gather is also worthy of study. The local non-DA neurons, especially GABAergic and glutamatergic cells, are involved in the providing the input to regulate the DA-regulated processes. Below we look into a bit more detail on these neuronal regulation systems, starting with the GABAergic neuronal system.

Inhibitory signals to midbrain DA (mDA) neurons are received from the GABAergic neurons located in the Ventral Tegmental Area (VTA), Substantia Nigra pars reticulata (SNpr) and Rostro Medial Tegmental nucleus (RMTg). Because of their association with dopamine neuron regulation as well as their proximity with the dopaminergic nuclei, these neurons were collectively coined dopaminergic neuron – associated GABAergic (D-GABA) neurons. Importantly, the D-GABA neuron subtypes share similar developmental origins and regulation in the ventral r1 (Achim et al, 2012; Lahti et al., 2016).

The GABAergic neurons of the SNpr project to the thalamus and superior colliculus, controlling voluntary- , eye- and reward- dependent movement. These neurons are associated with the DA neurons of the SNpc and inhibit the DA neurons with their axon collaterals. The neuronal density is low in the SNpr i.e. they are sparsely distributed. They create action potential spontaneously, without excitatory input (Zhou et al., 2011). The inhibitory GABAergic input from the SNpr to the thalamus and superior colliculus inhibits activation of movement, and SNpr GABAergic neuron inhibition thus allows movements. The GABAergic neurons in the SNpr are divided into subgroups based on neurochemical markers: parvalbumin, calretinin, nitric oxide synthase, and ephrins (Kelsom et al., 2013). Most of the SNpr GABAergic neurons are derived from the ventral r1, whereas others have an anterior origin (Achim et al., 2012). These SNpr neuron subtypes also have

differences in the expression and function of specific transcription factors (TFs) during their development (Lahti et al., 2016).

The GABAergic neurons of the RMTg are more involved in the control of phasic firing of the DA neurons, are activated by anterior brain regions signaling aversion, and are attached to functions such as motivated behavior and associative learning (Barrot et al., 2012). The RMTg GABAergic neurons are medium sized neurons expressing neurochemical markers, such as μ-opioid receptor, somatostatin and prepronociceptin. The RMTg inhibits the VTA and SNpc DA neurons during avoidance behavior. They also were found to express characteristic TFs (Lahti et al., 2016).

Addictive drugs, such as opioids, cannabinoids and benzodiazepines are thought to act on the VTA GABAergic neurons (Morales & Margolis, 2017). The inhibitory action of the VTA GABAergic neurons is carried out onto local DA neurons and these neurons are thought to process reward and reward expectations.

Glutamatergic neuron subtypes in the brainstem

The excitatory signals are provided by glutamatergic neurons and in the brainstem glutamatergic, neurons are found in the LDTg, IPN as well as locally in the VTA.

Dopamine neurotransmission is of two types based on firing rate: tonic and phasic. The former is based on basal neuronal activity and the latter is controlled by burst firing. Burst firing is essential for synaptic release of DA and consequently, plays a role in the reward and goal directed behaviors. In Lodge and Grace (2006), it is shown tonic input from the LDTg elicits burst firing. They observed that inactivation led to tonic release of dopamine as seen in a pacemaker. The LDTg bears neurons of three types: glutamatergic, GABAergic and cholinergic (Wang and Morales, 2009). The neurons from this nucleus send projections to the VTA to regulate the activation as well as the firing of the DA neurons. Most of these projections from the LDTg to the VTA are understood to be glutamatergic in nature (Lammel et al, 2012) and concurrently, optogenetic activation of the LDTg neurons has shown to corroborate the behavior consequences of these projections (Steidl, 2015). Hence, further supports the study of the LDTg and its surrounding architecture. Markers *Nkx6-1* and *Vsx2* are shown to be present in cells lateral to the DR. These cells are understood to form two different cell populations in the LDTg (Morello et al., 2020, Lahti et al, 2016) and consequently, are the markers used in this study.

Studies have identified glutamatergic neurons in the DA nuclei, which are responsible for local excitatory transmission (Morales and Root, 2014). In addition, the Interpeduncular nucleus (IPN), a nucleus below DA and RMTg, is understood to play a role in providing excitatory input to monoaminergic systems such as 5-HT (Lahti et al., 2016). The brainstem also bears glutamatergic neurons in many other regions, but their function has not been studied in detail.

Development of GABAergic and glutamatergic neurons in the ventral rhombomere 1

Tegmental GABAergic and glutamatergic neurons mainly originate in the rhombomere 1. Some originate dorsally. However, GABAergic neurons of the posterior SNpr (pSNpr), VTA, and RMTg, as well as glutamatergic neurons in the LDTg and IPN are born in ventral r1 (**Figure 2**). This region bears molecular similarity similar to the V2 domain of the spinal cord and hence, the name rV2. In the rV2 region, the proliferative progenitors that give rise to these neurons are marked by the expression of the TF *Nkx6-1*. The newly formed post-mitotic precursors in the rV2 are marked by distinct TFs i.e. GABA has *Gata2*, *Gata3*, *Tal1*, whereas the glutamatergic precursors express *Nkx6-1* and *Vsx2* as their TFs. The post-mitotic precursors expressing these TFs are intermixed in the rV2 regions close to (mx1) or more distal from (mz2) the proliferative progenitors in the ventricular zone (vz). These TFs push for the formation of these distinct nuclei like GABAergic SNpr and Glutamatergic LDTg/IPN. Thus, expression of particular TFs pushes the formation of various subtypes of GABAergic and glutamatergic neurons (Morello et al., 2020). However, the mechanism of how these TFs instruct these various subtype formation is still not clear.

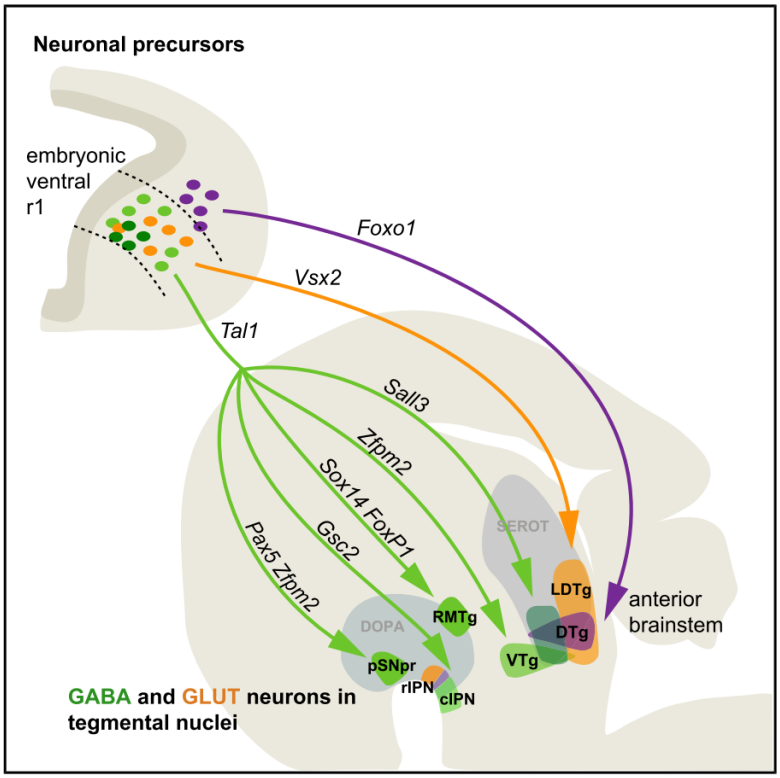


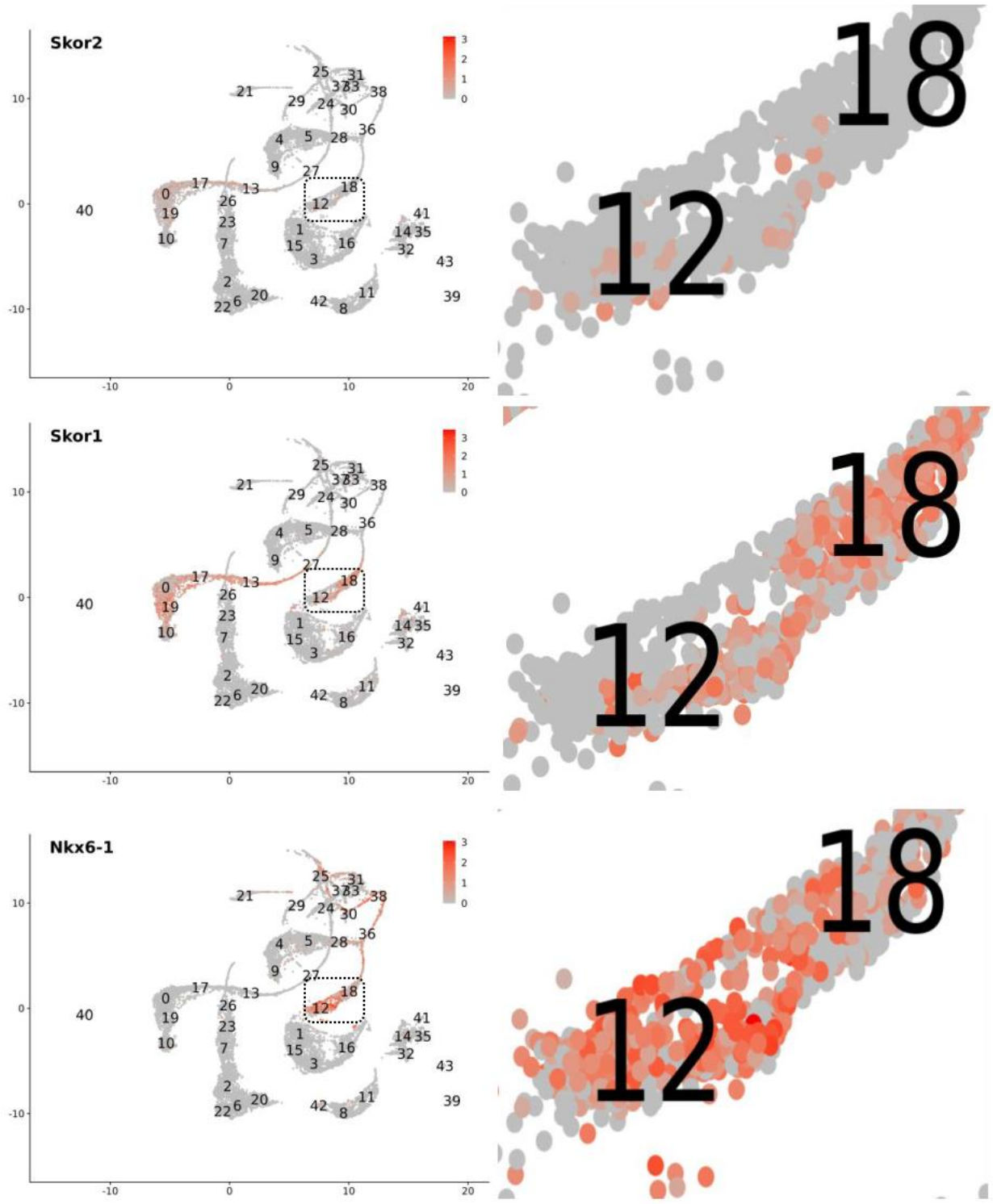
Figure 2. Origins of the brainstem GABAergic and glutamatergic neurons. A region of the ventral r1 gives rise to precursors of both GABAergic and glutamatergic neurons in brainstem nuclei **(**Source: Morello et al., 2020)

The structure and expression of the transcription factor *Skor2*

Skor2 protein is homologous to *Ski/Sno* family of transcriptional co-repressors. It is also known as *FUSSEL 18* Functional Smad suppressing element on chromosome 18 (Arndt et al., 2005). Another name, which represents it, is *Corl2,* because of its resemblance to corepressor for *LBX1* called *Corl1* (*FUSSEL 15* - Human analog). There are two structural domains in *Skor2:* Daschund Homology Domain (DHD) and SAND domain. These two domains are also seen in *Ski/Sno* family proteins and this family is associated with negative regulation of Transforming Growth Factor – β (TGF – β)/Bone Morphogenic Protein (BMP) signaling pathways through SMAD binding. Because of the sequence similarity, *Skor2* is understood to play a similar role inhibiting these pathways.

Previous research work (Nakatani et al, 2014; Wang et al, 2011) has focused on *Skor2* expression in the GABAergic Purkinje cells (PC) of the cerebellum. PC cells play a major role in providing the output from the cerebellum and is associated with functions such as movement control and posture. Degeneration of PCs leads to the rise of ataxic symptoms. *Skor2* expression was seen in PC lineage cells both during the development and during adulthood. *Skor2* mutants showed defects in cellular morphogenesis and PC maturation after birth. This suggested the *Skor2* is essential for PC differentiation during embryonic stages. Important for my study, *Skor2* was seen to affect GABAergic and glutamatergic phenotype in PCs (Nakatani et al, 2014). Specifically, the early GABAergic phenotype specification did not require *Skor2*, but maintenance of the GABAergic phenotype at later developmental stages required *Skor2*. At the same time, *Skor2* was shown to suppress glutamatergic phenotype in PCs. This suggests that *Skor2* could play a role as a co-activator of neuronal subtype features during PC differentiation.

Consistent with the role of *Skor2* in the PCs developing from the dorsal r1, data from single cell RNA sequencing of E13.5 mice has shown the presence of *Skor2* transcripts in dorsal r1 -derived GABAergic precursors that migrate to the ventral r1 during the brainstem development (Morello et al., 2020). Important for this Master's thesis work, some *Skor2* expressing cells were also detected in the ventral r1 (rV2) - derived glutamatergic neuron precursors (Figure 3). Thus, *Skor2* does not appear to exclusively mark GABAergic precursors. This is consistent with the expression of *Skor1*, a TF related to *Skor2,* both in the dorsally derived GABAergic and in the rV2 derived glutamatergic precursors (Morello et al., 2020) (**Figure 3**).


Skor2
Skor1
Nkx6-1
18
12

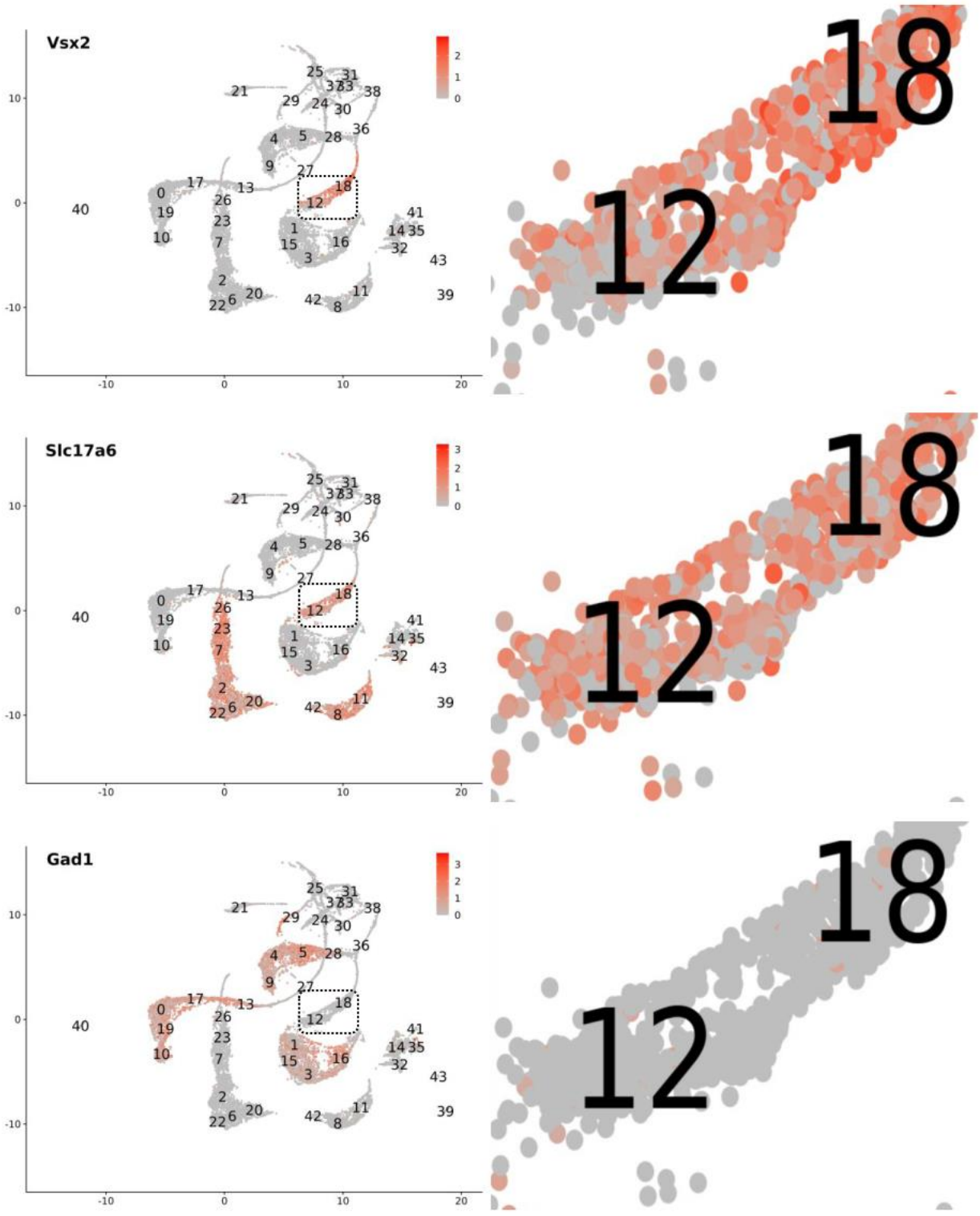


Figure 3: **Visualization of expression of *Skor2* and selected other marker genes in brainstem single cell mRNA sequencing data.** UMAP of E13.5 mice showing the expression of Glutamatergic markers: *Vglut2* (*Slc17a6*), *Vsx2* (*Chx10*), *Nkx6-1*; GABAergic marker: *Gad1, Skor1* (homology to *Skor2)* and *Skor2*. *Skor2* is found in the dorsally derived GABAergic precursors (expression in the early Purkinje

cells derived from the dorsal rhombomere 1) and in some rV2 derived glutamatergic precursors (dotted box). The right side images are close-up of the area of interest (rv2 domain) for each respective marker. (Morello et al., 2020) and gene expression database at tegex.helsinki.fi.

Aims

Aim 1: Characterization of *Skor2* expression in the embryonic brainstem precursors and tegmental neurons derived from them

Skor2 positive cells were observed in the rV2 derived glutamatergic precursor clusters in E13.5 single cell mRNA sequencing data. I hypothesize that *Skor2* is expressed in a subset of glutamatergic neuronal precursors in the embryonic rV2 tissue. I also hypothesize that, at later developmental stages, these glutamatergic precursors move to the LDTg region. To test these hypotheses, I will compare the tissue distribution of *Skor2* expression with glutamatergic markers *Nkx6-1* and *Vsx2*, and address to what extent *Skor2* is co-expressed along with these markers in the embryonic ventral r1 and in the mature tegmental nuclei, including the LDTg and other brainstem regions.

Aim 2: Characterization of the function of *Skor2* in brainstem glutamatergic neuron development

Skor2 has been shown to regulate neuronal differentiation in other contexts. I hypothesize that *Skor2* is important for differentiation of the rV2 derived glutamatergic neurons. To test this hypothesis, I will study rV2 glutamatergic precursor development and the glutamatergic brainstem nuclei in the *Skor2* knockout mice.

Materials and Methods

Mice

The background of the mice used in this study are of the ICR strain. This strain of mouse is albino outbred strain and was selected at the Institute of Cancer Research, USA (Chia et al. 2005, Rice & Brien 1980). E0.5 is designated as the noon of the day the vaginal plug happened. The *Skor2* $^{GFP/+}$ mice were generated by introducing a green fluorescent protein (*GFP*) open reading frame after the initiation codon of the *Skor2* gene. The process involves formation of a *Skor2 targeting* vector using p*GFP*-neo-DT-A (**Figure 4**). The *Skor2*-null mice were generated by homologous recombination of the Wild type ICR mice with the vector and the mutation was confirmed through Southern blotting. These mice express *GFP* at the location of *Skor2* positive cells, while maintaining function of *Skor2 repressor*. *Skor2* $^{GFP/GFP}$ homozygous knockouts were created by crossing the *Skor2* $^{GFP/+}$ heterozygous mutants with each other. These homozygote mutants were selected using PCR and subsequent gel electrophoresis.

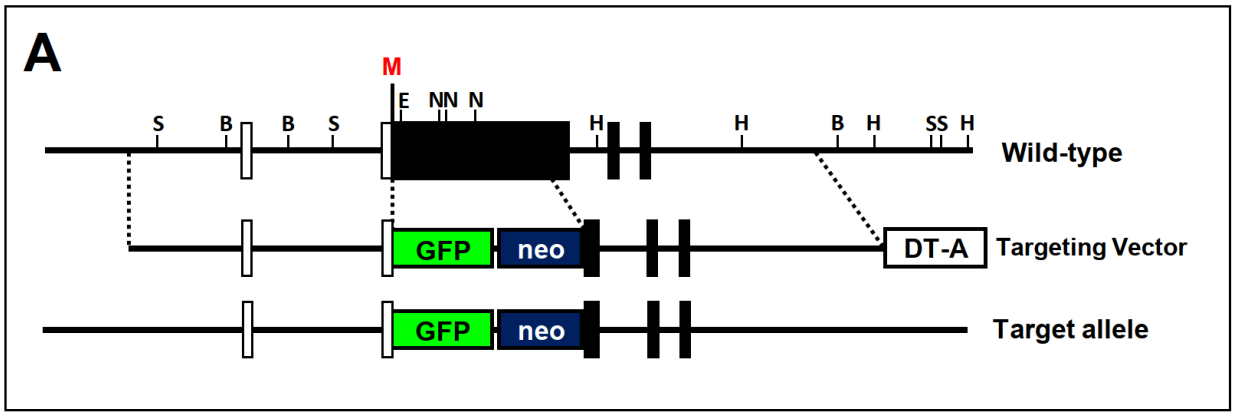


Figure 4: How the *Skor2* allele is replaced with *GFP*. *GFP* cDNA in substituted instead of the Ski domain. Black – Coding region, White – Non-coding region. Initiation codon bears the following: M. S: ScaI; B, BamHI; E: EcoRV; N: NotI; H: HindIII (Source: Nakatani et al, 2014)

Histology

Fixation

The whole embryo (E11.5-E13.5) and whole brain (E15.5, E18.5) cassettes were placed on a shaker with a solution of 4% PFA (paraformaldehyde) overnight. The PFA is replaced over the next two days and left on the shaker. Then, the sample is ready for tissue processing and paraffin embedding.

Tissue processing

Tissue processing is done via Leica Biosystems Fully enclosed tissue processor. The processor allows the infiltration of the paraffin via treatment with various solvents. This is required as the wax is hydrophobic and immiscible with water. The process starts with the whole embryo/whole brain sample laden cassette immersed in MilliQ water and then followed by multiple changes of ethanol (dehydrating agent) & xylene (clearing agent). The processing is allowed to run overnight, which facilitates the tissue embedding the next day.

Embedding

The embryo sample is taken from the cassette and placed in a tray of hot paraffin. The sample is positioned to facilitate the correct angle for sectioning. Then, the cassette is placed on top and then whole structure is carefully moved onto a cryoplate, which is set at -1°C. This allows the paraffin to solidify and trap the sample in a desired position. We can separate the sample from the tray after a minimum of 30 minutes. The paraffin-embedded sample is now ready to be sectioned.

Sectioning

In this step, the paraffin-embedded sample is placed in a microtome and cut of shavings of the wax with the sample in them. These sections are then placed on adhesive slides and left overnight in a 32°C incubator. The slides are then taken out and placed on a heat block set at 70°C for around a minute. This is to remove the excess paraffin, leaving behind mostly the sample. These slides can be used immediately for staining or can be stored away at +4°C. Example of the cut is shown (dotted line) below. The purple region denotes the rhombomere1 (r1).

Figure: 5. E11.5 mouse embryo (Source: Allen Mouse Brain Atlas) Dotted line represents the angle of sectioning. Purple region denotes the rhombomere1

Immunohistochemistry

Skor2 was studied through the immunohistochemistry method. Slides are brought out from +4°C beforehand to let them reach room temperature before staining.

Deparaffination/Rehydration

The aim is to rehydrate the samples, so that we can return the samples back from its dormancy. This is done before the staining protocol, as incomplete removal of paraffin can affect staining efficiency.

- Xylene 4 x 5 mins (the last xylene fresh each time)
- Abs. Ethanol 3 x 2 mins (the last ethanol fresh each time)
- 94% Ethanol 2 x 2 mins
- 70% Ethanol 1 x 2 mins
- 50% Ethanol 1 x 2 mins
- MQ 2 x 1 min

Antigen retrieval

This step allows breaking the crosslinks protecting the protein from deterioration. Crosslinks served their purpose earlier in storage. Now we need to return the sample to more native state by heat induced epitope retrieval (HIER).

The slides were then moved to a glass tray, holding 250ml 0.01 M sodium citrate buffer pH 6.0. The setup was then moved to a microwave, where it was blasted at high for 3 minutes and then for 9 minutes at a lower setting. They setup is allowed to cool down for handling, making sure not to let

it sit for over 30 minutes. The longer they slides are in the hot buffer, the chances are the protein targets may be harmed. Then the slides are washed in PBS for 5minutes on the shaker, which is then followed by the Permeabilization step.

Permeabilization

This step allows better penetration of the antibody solution and is performed by washing the slides in a detergent buffer solution of 0.3% PBST (Triton-X in PBS) for 45 minutes on the shaker. This is followed by just rinsing twice will MilliQ water, to get rid of the excess detergent.

Blocking:

Before adding the antibodies, we want to prevent any non – specific binding to tissue. Serum is generally used for such purpose and their origin is linked to which secondary antibodies are used. Since the secondary antibodies are from donkey, the serum is also from the same species. Here the slides are taken from the box and placed in a humidifying chamber to avoid drying up. 300µl of 10% Donkey serum in 0.3% PBST is pipetted onto these slides. This setup is left alone for 1 hour.

Primary antibody:

During the blocking time, antibody dilutions are prepared in a solution of 5% donkey serum + 0.1%Triton X-100 in PBS. The antibodies are kept on ice, while making the dilutions. The table below shows which antibodies were used and in what dilutions. After the dilutions were prepared, the blocking solution is removed from the slides. 300µl of the appropriate antibody dilutions were added. Above process is performed in the humidifying chamber and then left closed at 4°C over 2 nights.

Table 1. The primary and secondary antibodies used for paraffin sections.

	Host species	**Antigen name**	**Supplier**	**Product code**	**Dilution**
Primary antibodies	Rabbit	*GFP*	Abcam	ab290	1:2000
	Mouse	*Nkx6-1*	DSHB	F55A10	1:1000
	Sheep	*Vsx2*	Abcam	ab16141	1:500
	Goat	ChAT	Chemicon	AB144P	1:100
	Mouse	Fog2	Santa Cruz Biotechnology	sc-398011	1:500
	Rabbit	FoxO1	Cell Signaling Technology	mAb #2880	1:500
	Mouse	TH	Chemicon	MAB318	1:600
	Goat	5-HT	ab66047	ab66047	1:500
	Rabbit	5-HT	Immunostar	20080	1:500

Secondary antibody:

After the primary antibody incubation, the slides were removed from the chamber and washed three times for 5 minutes in a 0.1% PBST solution, on a shaking platform. As was done before, the secondary antibody dilutions were prepared in *DAPI*+0.3% PBST (1:10000). The dilutions and the antibodies should be kept away from light sources, as much as possible. This is done to avoid bleaching of the fluorophores attached to the antibodies, which give us the fluorescence. The dilutions are then added (300µl) to the slides in the chamber. The slides are kept away from light as much as possible and then allowed to rest at room temperature for 5 hours.

	Name	Fluorescent dye	Supplier	Product code	Dilution
Secondary antibodies	Donkey-anti-mouse IgG	Alexa Fluor 568 (red)	Thermo Fisher Scientific	A10037	1:400
	Donkey-anti-mouse	Alexa Fluor 488 (green)	Thermo Fisher Scientific	A21202	1:400
	Donkey-anti-rabbit	Alexa Fluor 488 (green)	Thermo Fisher Scientific	A21206	1:400
	Donkey-anti-sheep IgG	Alexa Fluor 568 (red)	Thermo Fisher Scientific	A21099	1:400
	Donkey-anti-goat IgG	Alexa Fluor 568 (red)	Thermo Fisher Scientific	A11058	1:400
	Donkey-anti-rabbit IgG	Alexa Fluor 568 (red)	Thermo Fisher Scientific	A10042	1:400

The slides were then removed from the chamber and washed in a solution of 0.1% PBST for 5 minutes on a platform shaker. The solution is then replaced with PBS and the washes are repeated three times for 5 minutes each. The Slides are then mounted with coverslips using Fluorsave and then stored at +4°C, away from light.

Imaging

The slides were studied using Olympus BX63 Microscope (DP72 Camera) and this allowed us to take images from them. The images taken were merged and processed using Fiji ImageJ 1.52 (64 bit)

Quantification/Cell counting and Statistical analysis

The quantification was also done with the Fiji Image J 1.52 (64 bit), using its manual counter. The data was accumulated in Excel and utilized to show the data in chart form. Student t-tests were performed to show the statistical significance of the data accumulated.

Results

Skor2 is expressed in post-mitotic rV2 precursors in the mz2 along with *Nkx6-1* at E11.5-E13.5

Skor2 positive cells were seen in cell groups expressing the rV2 glutamatergic markers *Nkx6-1* and *Vsx2* in the E13.5 single cell RNA sequencing data (Morello *et al*., 2020). Therefore, I compared the expression of *Skor2* and *Nkx6-1* in the embryonic ventral r1 tissue. To visualize *Skor2* expression, I used IMHC to detect *GFP* and *Nkx6-1* expression in coronal sections of E11.5-E13.5 *Skor2* $^{GFP/+}$ embryos. I found that almost all *GFP* expression from the *Skor2* ^{GFP} allele was in *Nkx6-1* positive (*Nkx6-1*$^{+}$) cells, but not all *Nkx6-1*$^{+}$ cells expressed *GFP* (**Figure 6**). The majority of *Nkx6-1* cells were found in the proliferative progenitors of the ventricular zone (vz) and in the post-mitotic precursors of the mantle zone 2 (mz2) region, while the *Skor2* positive cells occupied both post mitotic mz1 and mz2 regions. The quantification of Skor2 positive and *Skor2+Nkx6-1* double positive cells in the anterior and posterior brainstem is shown in **Figure 8.**

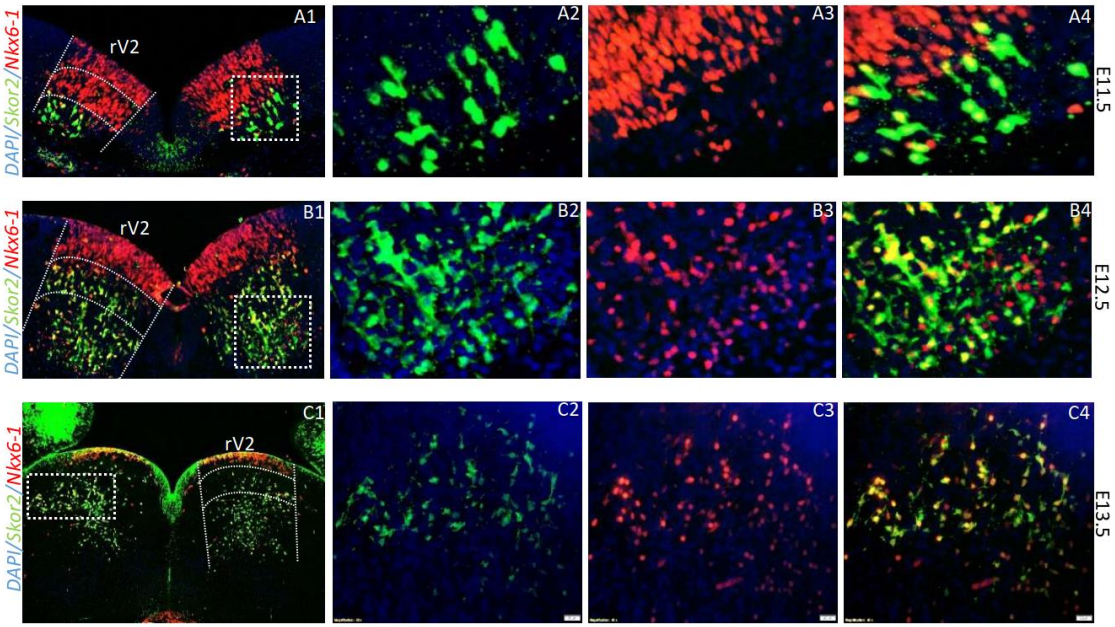


Figure 6. Analysis of the expression of *Skor2* and *Nkx6-1* in the rhombomere1 of early embryonic mouse brain

A1-A4: E11.5, B1-B4: E12.5, C1-C4: E13.5; A1: Analysis of *Skor2* and *Nkx6-1* expression at E11.5. The square box represents the area of interest shown in A2-A4. The rV2 domain is marked with lines. A2: Close-up of *Skor2* expression. A3: Close-up of *Nkx6-1* expression. A4: Merge of *Skor2* and *Nkx6-1* expression. B1: Analysis of *Skor2* and *Nkx6-1* expression at E12.5. The square box represents the area of interest shown in B2-B4. The rV2 domain is marked with lines. B2: Close-up of *Skor2* expression. B3: Close-up of *Nkx6-1* expression. B4: Merge of *Skor2* and *Nkx6-1* markers in the area of interest. C1: Analysis of *Skor2* and *Nkx6-1* expression at E13.5. The square box represents the area of interest. The rV2 domain is marked with lines. C2: Close-up of *Skor2* expression. C3: Close-up of *Nkx6-1* expression. C4: Merge of *Skor2* and *Nkx6-1* expression. DAPI is used for nuclear staining in all the images. Scale bar: 50 µm (The rV2 domain is indicated with a line and divided into ventricular zone - vz, mantle zone 1 – mz1, mantle zone – mz2)

Skor2 has minimal co-expression with _Vsx2_, the latter predominantly marking the rV2 mz1 at E11.5-E13.5

Vsx2/*Chx10* is another TF marking specifically the post-mitotic glutamatergic neuron precursors in the rV2 (Lahti et. al 2016, Morello et al., 2020, Figure 3). To analyze whether the *Skor2* positive precursors also express *Vsx2*/*Chx10*, I performed IMHC with markers *GFP* and *Vsx2*. The *GFP* marker tags *Skor2* positive cells, as the mice used were $Skor2^{GFP/+}$. Analyzing the staining, I observed that *Vsx2* positive cells were mostly confined to the mz1, whereas the *Skor2* positive cells were found in the mz2 (**Figure 7**). There were some cells expressing both *Vsx2* and *Skor2* at the border of mz1 and mz2. However, in comparison to Nkx6-1, the number of double positive cells was lower. The *Skor2* positive cells were confined to the rv2 domain, as observed in the *Nkx6-1* staining. **Figure 8** shows the quantification data in the anterior and posterior brainstem of markers *Skor2, Nkx6-1* and *Vsx2* together. This shows the clear difference in the number of *Skor2* cells expressing *Nkx6-1* more than them expressing *Vsx2.*

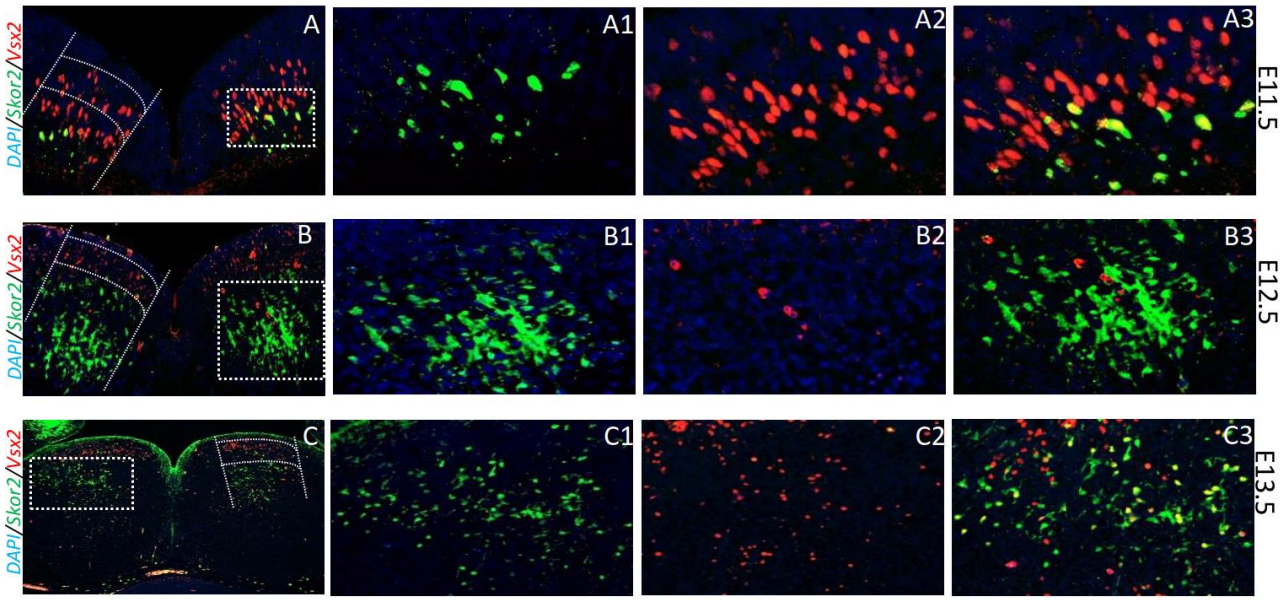


Figure 7. Analysis of expression of *Skor2* and *Vsx2 (Chx10)* in rhombomere1 of early embryonic mouse brain

A1-A4: E11.5, B1-B4: E12.5, C1-C4: E13.5; A1: Analysis of *Skor2* and *Vsx2* expression at E11.5. The square box represents the area of interest shown in A2-A4. The rV2 domain is marked with lines. A2: Close-up of *Skor2* expression. A3: Close-up of *Vsx2* expression. A4: Merge of *Skor2* and *Vsx2* expression. B1: Analysis of *Skor2* and *Vsx2* expression at E12.5. The square box represents the area of interest shown in B2-B4. The rV2 domain is marked with lines. B2: Close-up of *Skor2* expression. B3: Close-up of *Vsx2* expression. B4: Merge of *Skor2* and *Vsx2* markers in the area of interest. C1: Analysis of *Skor2* and *Vsx2* expression at E13.5. The square box represents the area of interest. The rV2 domain is marked with lines. C2: Close-up of *Skor2* expression. C3: Close-up of *Vsx2* expression. C4: Merge of *Skor2* and *Vsx2* expression. DAPI is used for nuclear staining in all the images. Scale bar: 50 µm (The rV2 domain is indicated with a line and divided into ventricular zone - vz, mantle zone 1 – mz1, mantle zone – mz2)

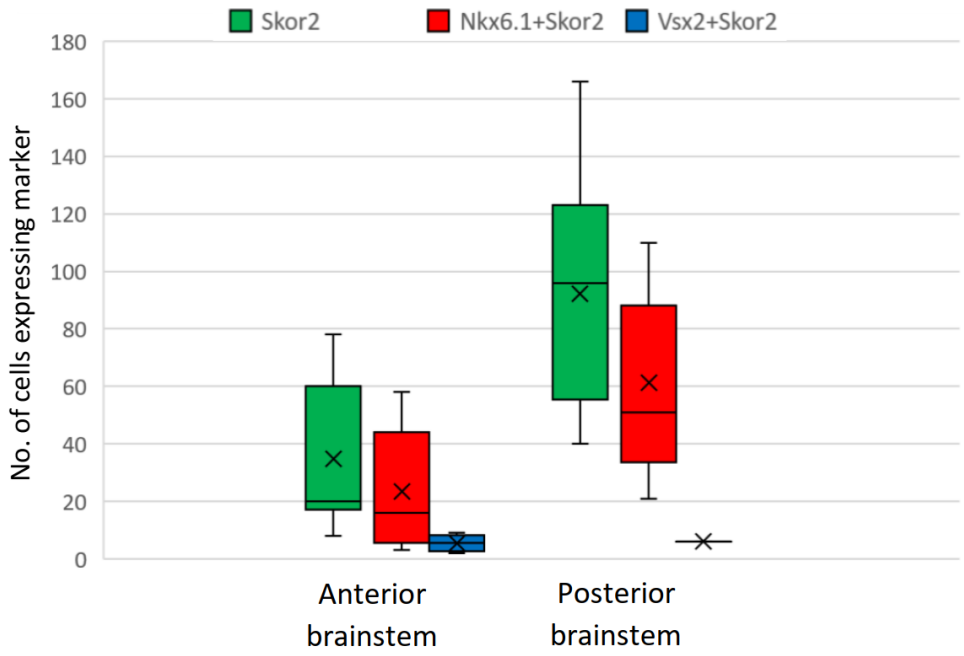


Figure 8. Quantification of *Skor2* positive and *Skor2+Nkx6-1* double positive cells in E13.5 brainstem. *Skor2* positive cells versus coexpression of *Skor2* and *Nkx6-1* in the anterior brainstem ($p>0.05$ – Student t-test) and similar comparison between the populations in the posterior brainstem ($p<0.05$ – Student t-test). Comparison of the anterior and posterior Brainstem cell populations via student t-test yielded: *Skor2+ve* cells ($p<0.05$), *Skor2* and *Nkx6-1* double positive cells ($p<0.05$). More embryos were needed for a more quantitative analysis of Vsx2 staining. Data provided here is just to elucidate there are more *Skor2* positive cells expressing *Nkx6-1* than *Vsx2.*

Skor2 is not expressed in the LDTg along with _Nkx6-1_, but shows expression in a region more ventral

Next, I wanted to examine whether the *Skor2* positive cells that were observed in the rv2 domain, would give rise to the LDTg glutamatergic neurons at the later embryonic stages. Coronal sections of E15.5 and E18.5 *Skor2*$^{GFP/+}$ mice were used for IMHC with markers *GFP* and Nkx6-1. In contrast to my hypothesis, no *Skor2* expression was detected in the *Nkx6-1* positive LDTg neurons. Instead, the staining showed *Skor2* positive cells occupying a distinct position more ventrally and away from the LDTg (**Figure 9)**. This cluster still had a glutamatergic identity, as suggested by *Nkx6-1* coexpression. We also the quantification data for *Skor2* and *Skor2+Nkx6-1* double positive cells in the brainstem (**Figure 10**).

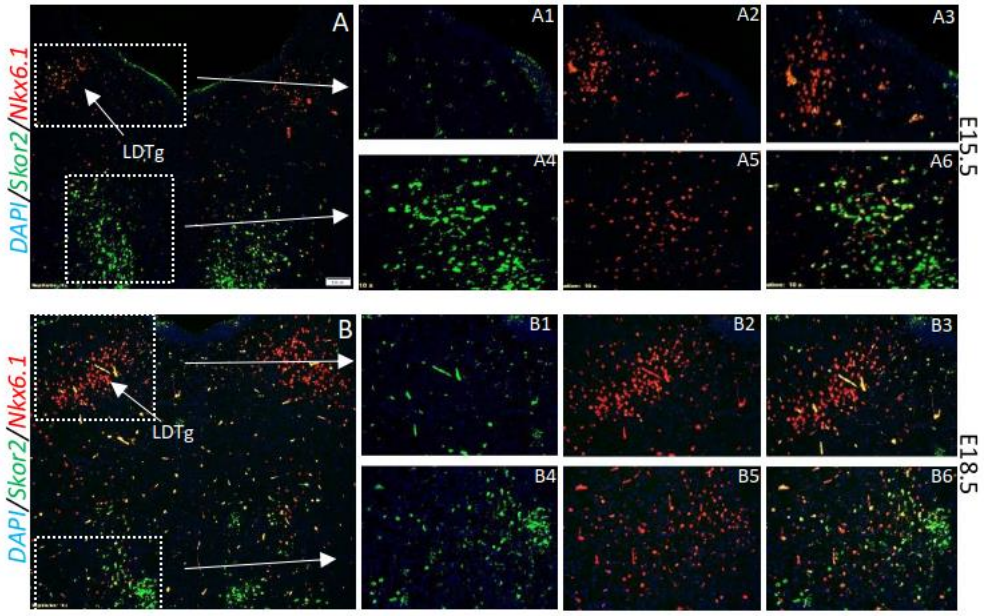


Figure 9. Analysis of expression of *Skor2* and *Nkx6-1* in rhombomere1 of later embryonic mouse brain (E15.5 & E18.5)

A-A6: E15.5, B1-B6: E18.5. A: Analysis of *Skor2* and *Nkx6-1* expression at E15.5 rhombomere1. The dorsal square box represents the LDTg, area of interest shown in A1-A6. A1: Close-up of *Skor2* expression. A3: Close-up of *Nkx6-1* expression. A4: Merge of *Skor2* and *Nkx6-1* expression. The ventral area of interest is shown in A4-A6. A4: Close-up of *Skor2* expression. A5: Close-up of *Nkx6-1* expression. A6: Merge of *Skor2* and *Nkx6-1* expression. B: Analysis of *Skor2* and *Nkx6-1* expression at E18.5 rhombomere1. The dorsal square box represents the LDTg, area of interest shown in A1-A6. A1: Close-up of *Skor2* expression. A3: Close-up of *Nkx6-1* expression. A4: Merge of *Skor2* and *Nkx6-1* expression. The ventral area of interest is shown in A4-A6. A4: Close-up of *Skor2* expression. A5: Close-up of *Nkx6-1* expression. A6: Merge of *Skor2* and *Nkx6-1* expression. DAPI is used for nuclear staining in all the images. Scale bar: 50 µm

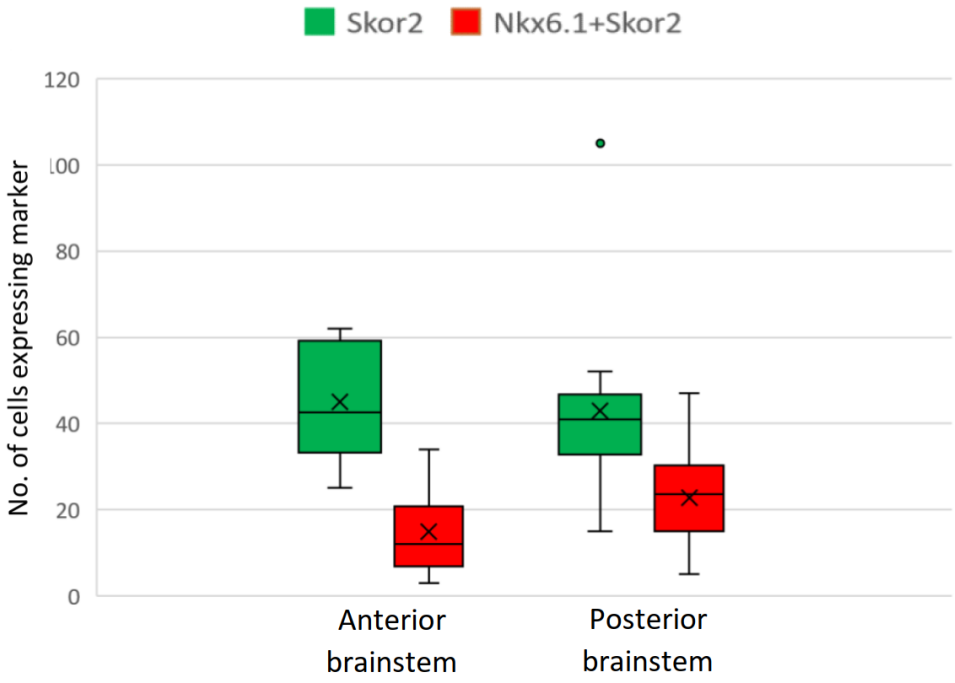


Figure 10. Quantification of *Skor2* positive cells in the brainstem of E18.5 mouse.

A: *Skor2* positive cells versus co-expression of *Skor2* and *Nkx6-1* in the anterior ($p<0.05$ – Student t-test) and posterior brainstem cell populations ($p<0.05$ – Student t-test). Comparison between the Anterior and Posterior Brainstem cell populations via student t-test yielded: *Skor2+ve* cells ($p>0.05$), *Skor2* and *Nkx6-1* double positive cells ($p<0.05$).

Skor2 is predominantly expressed in _Vsx2_ negative cells at E15.5 and E18.5

I also compared the expression of *Skor2* and *Vsx2* in the LDTg and other brainstem regions. This was done with IMHC staining of markers *GFP* and *Vsx2*. The staining showed similar results as observed with *Nkx6-1* co-staining (**Figure 11**). However, the difference being the number of cells that express *Skor2* and *Vsx2* together was lower in number. The LDTg did not bear any *Skor2* positive cells. Similar to analyses with *Nkx6-1*, the *Skor2* positive cells were found to be in a cluster markedly lower. Quantification data was not available for *Vsx2* costaining, due to inconsistent results with the antibody targeting the marker in question.

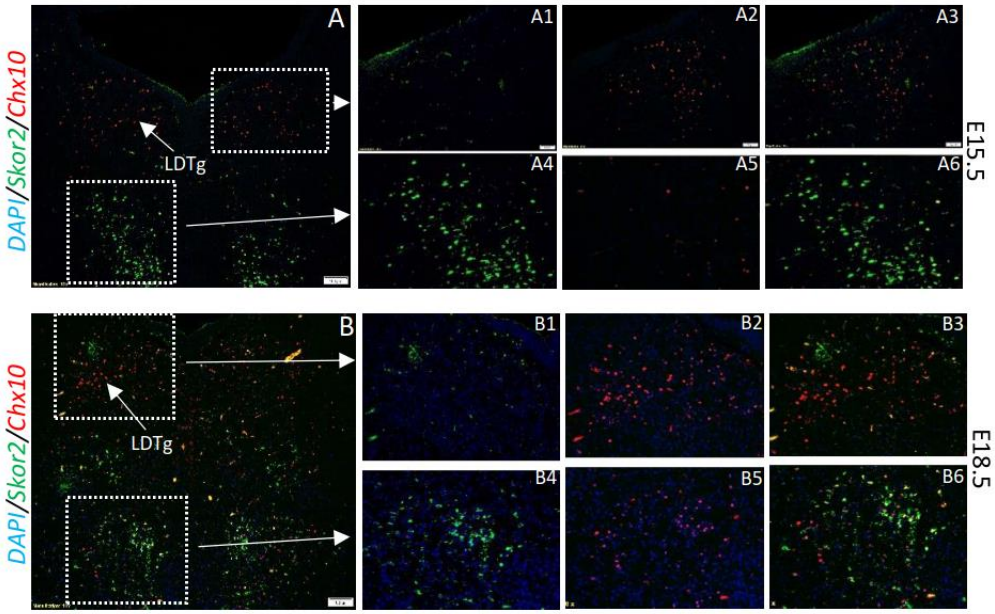


Figure 11. Expression analysis of *Skor2* and *Vsx2 (Chx10)* in rhombomere1 of E15.5 and E18.5 mouse embryo

A-A6: E15.5, B1-B6: E18.5. A: Analysis of *Skor2* and *Vsx2* expression at E15.5 rhombomere1. The dorsal square box represents the LDTg, area of interest shown in A1-A6. A1: Close-up of *Skor2* expression. A2: Close-up of *Vsx2* expression. A3: Merge of *Skor2* and *Vsx2* expression. The ventral area of interest is shown in A4-A6. A4: Close-up of *Skor2* expression. A5: Close-up of *Vsx2* expression. A6: Merge of *Skor2* and *Vsx2* expression. B: Analysis of *Skor2* and *Vsx2* expression at E18.5 rhombomere1. The dorsal square box represents the LDTg, area of interest shown in B1-B6. B1: Close-up of *Skor2* expression. B2: Close-up of *Vsx2* expression. B3: Merge of *Skor2* and *Vsx2* expression. The ventral area of interest is shown in B4-B6. B4: Close-up of *Skor2* expression. B5: Close-up of *Vsx2* expression. B6: Merge of *Skor2* and *Vsx2* expression. DAPI is used for nuclear staining in all the images. Scale bar: 50 µm

Anatomical characterization of *Skor2* expressing cells in the posterior brainstem: identification of *Skor2* positive neurons between the VTg and DTg

My studies above showed that *Skor2* is not expressed in the LDTg, but in a brainstem region ventral to it. I next wanted to define, with gene expression landmarks, the area of brainstem where the *Skor2* expressing cells were located. The sections of the rhombomere 1 were first characterized using reference stains such as Tyrosine Hydroxylase (*TH*) and Serotonin (*5-HT*). Studies of embryos at E12.5, E15.5 and E18.5 showed that the *Skor2* expressing cells end up in a region ventrolateral to the *5-HT* positive DR and medial to the *TH* positive Locus coeruleus (LC) (**Figure 12**).

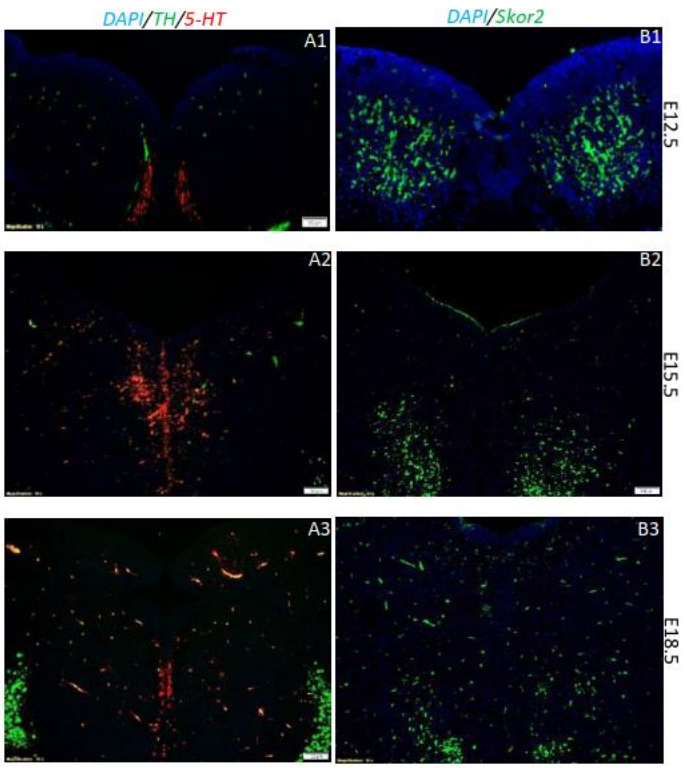


Figure 12. Anatomical characterization of *Skor2* positive cells in comparison to cells expressing TH and 5-HT markers. A1 – A3: Expression profile of *TH* and *5-HT* markers. B1 – B3: Corresponding expression of *Skor2* A1: *TH* and *5-HT* expression in E12.5 rhombomere 1. B1: *Skor2* expression in relevant E12.5 embryo brainstem. A2: Expression of *TH* and *5-HT* in rhombomere 1 of E15.5. B2: rhombomere 1 *Skor2* expression in E15.5 embryo. A3: E18.5 rhombomere 1 expression pattern of markers *TH* and *5-HT.* B3: Example area of interest in rhombomere 1 of E18.5 showing *Skor2* expression.

Originally, we had chosen *ChAT* (Choline Acetyl transferase) as a marker to study the expression of *Skor2* in cholinergic neurons of rhombomere 1. However, *ChAT* positive cells only show up in later embryonic stages and are nowhere near the *Skor2* positive cells (Image not shown). Hence, *ChAT* was not an appropriate marker to study *Skor2* expression in rhombomere 1.

As we observed in the immunostaining of *Skor2*, the TF's expression was observed in a region ventral to the LDTg. This area could be studied in comparison to other markers expressed in the region, namely markers expressing the in the Dorsal and the Ventral Tegmentum of Gudden (DTg and VTg). The DTg nucleus has cells that express *FoxO1* marker and the *Fog2* represents the VTg region (Morello et al., 2020). The appropriate sections were chosen to undergo immunostaining with the following sets: *GFP* & *Fog2* and *FoxO1* & *Fog2*. The results of these staining's demonstrate *Skor2* expression in an area bordering both the *FoxO1* and *Fog2* (**Figure 12**). These cluster of cells are not defined, to my knowledge, and may provide new avenues for study.

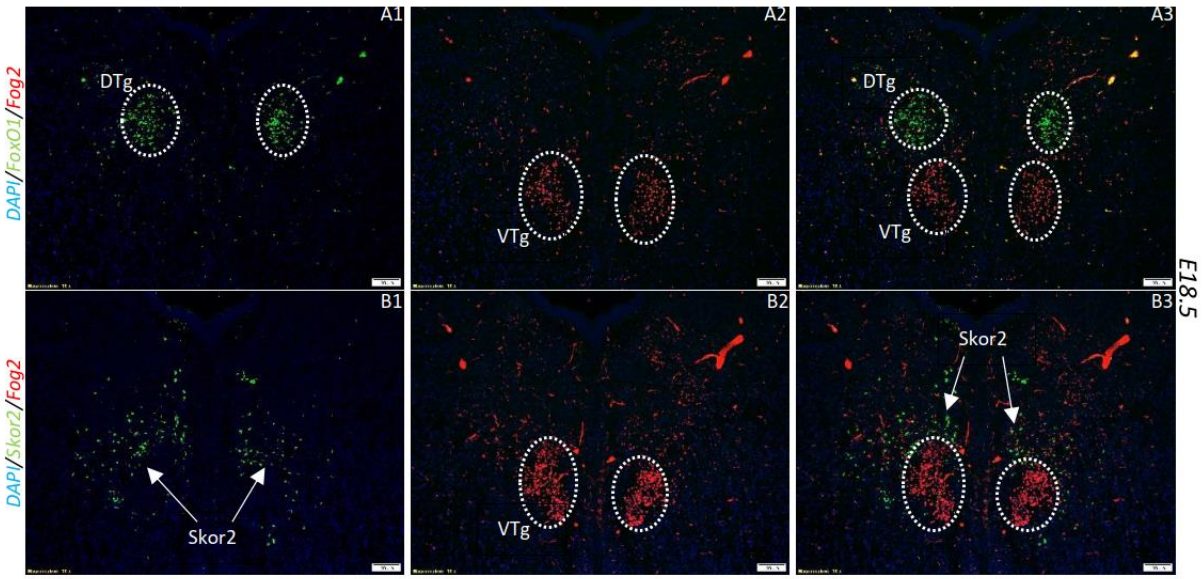


Figure 12. Anatomical comparison of *Skor2* positive cells with DTg (*FoxO1*) and VTg (*Fog2*) markers at E18.5. A1 – A3: Expression pattern of markers *FoxO1* and *Fog2* in E18.5 rhombomere 1. A1: *FoxO1* expression in E18.5 rhombomere 1. A2: *Fog2* expression analysis. A3: Merge of *FoxO1* and *Fog2* expression. B1 – B3: *Skor2* and *Fog2* expression in parallel sections of same embryo. B1: *Skor2* staining of E18.5 rhombomere 1 . B2: *Fog2* expression at rhombomere 1. B3: Merge of *Skor2* and *Fog2* stainings.

<u>*Skor2* deficient mice do not seem to have changes in the brainstem cell types</u>

This section deals with the second part of the experiment, where I studied the effects of knocking out *Skor2*. This allows us to understand the how important *Skor2* protein is for the survival of the brainstem neurons. Here I studied the E13.5 $Skor2^{GFP/+}$ and $Skor2^{GFP/GFP}$ embryos side by side through IMHC, using markers for *GFP* and *Nkx6-1*. In the $Skor2^{GFP}$ allele, the *Skor2* is replaced completely by *GFP* (A-A3), resulting in a functionally null allele of the gene. The *GFP* insertion allows us to show where the *Skor2* expression exists and compare *Skor2* expression in heterozygous and homozygous mutants (B-B3). As seen below, I observed no clear changes during the early embryonic stages between the heterozygote and the homozygote (**Figure 13**). This argues against an essential nature of *Skor2* in embryonic stage.

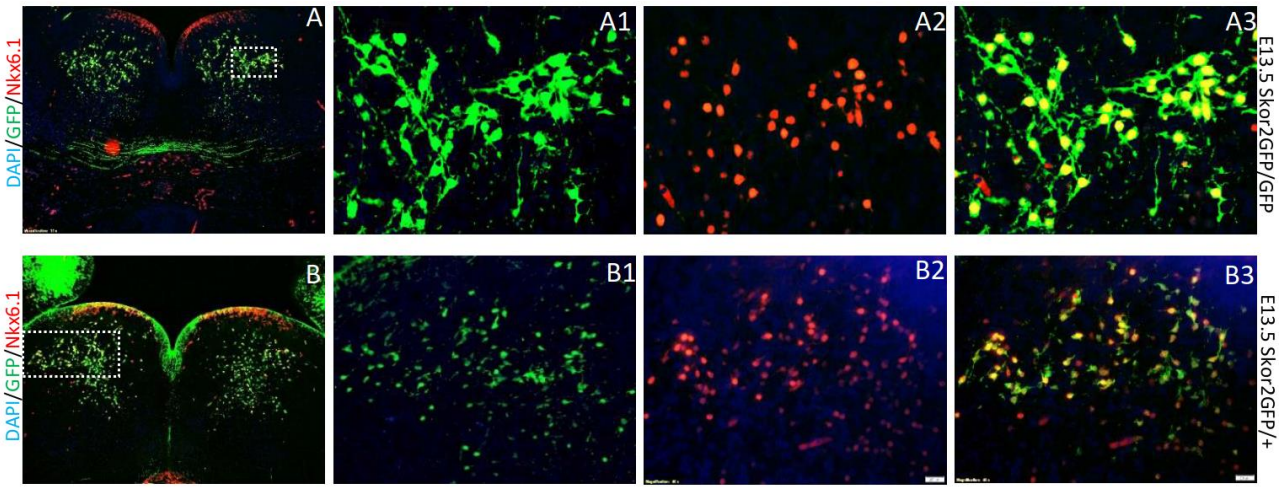


Figure 13. Analysis of the expression of *Skor2* and *Nkx6-1* in the rhombomere1 of early embryonic mouse brain of heterozygous and homozygous *Skor2* mutants.

A-A3: *Skor2GFP/GFP* E13.5. A: Analysis of *Skor2* and *Nkx6-1* expression at E13.5. The square box represents the area of interest. The rV2 domain is marked with lines. A1: Close-up of *Skor2* expression. A2: Close-up of *Nkx6-1* expression. A3: Merge of *Skor2* and *Nkx6-1* expression. B-B3: $Skor2^{GFP/+}$ E13.5. A: Analysis of *Skor2* and *Nkx6-1* expression at E13.5. The square box represents the area of interest. The rV2 domain is marked with lines. B1: Close-up of *Skor2* expression. B2: Close-up of *Nkx6-1* expression. B3: Merge of *Skor2* and *Nkx6-1* expression. DAPI is used for nuclear staining in all the images. Scale bar: 50 µm

Similarly, I studied the expression profile of *Skor2* in E18.5 *Skor2*$^{GFP/+}$ and *Skor2*$^{GFP/GFP}$ embryos (**Figure 14**). This was done by IMHC with markers *GFP* and *Nkx6-1*. The LDTg region again remained devoid of *Skor2* presence. I detected no changes in the expression of *Nkx6-1* LDTg region. Moreover, the ventral region of *Skor2* expression between VTg and DTg was intact and expresses the same markers. Anatomically, the *Skor2* positive cells remained in the same region. And similarly there did not seem ato be any changes observed with the glutamatergic marker *Nkx6-1*. This suggests the glutamatergic neurons retaining their normal function and structure. This suggests that *Skor2* is not essential for brainstem neuron maturation at late embryonic stages.

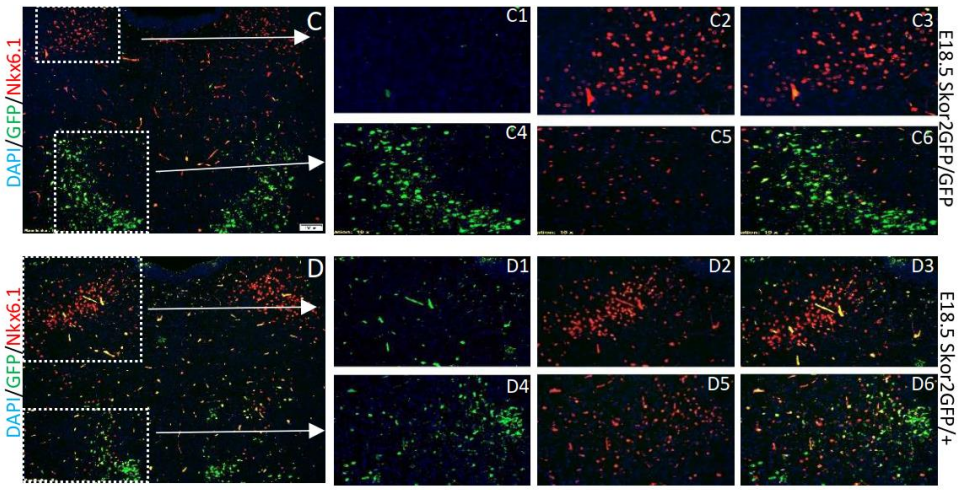


Figure 14. Expression analysis of *Skor2* and *Nkx6-1* in rhombomere1 of E18.5 *Skor2*$^{GFP/+}$ and E18.5 *Skor2*$^{GFP/GFP}$ mouse embryo.

C-C6: E18.5 *Skor2 GFP/GFP*, D-D6: E18.5 *Skor2 GFP/+*. C: Analysis of *Skor2* and *Nkx6-1* expression at E18.5 rhombomere1. The dorsal square box represents the LDTg, area of interest shown in C1-C3. C1: Close-up of *Skor2* expression. C2: Close-up of *Nkx6-1* expression. C3: Merge of *Skor2* and *Nkx6-1* expression. The ventral area of interest is shown in C4-C6. C4: Close-up of *Skor2* expression. C5: Close-up of *Nkx6-1* expression. C6: Merge of *Skor2* and *Nkx6-1* expression. D: Analysis of *Skor2* and *Nkx6-1* expression at E18.5 *Skor2 GFP/+* rhombomere1. The dorsal square box represents the LDTg, area of interest shown in D1-D3. D1: Close-up of *Skor2* expression. D2: Close-up of *Nkx6-1* expression. D3: Merge of *Skor2* and *Nkx6-1* expression. The ventral area of interest is shown in D4-D6. D4: Close-up of *Skor2* expression. D5: Close-up of *Nkx6-1* expression. D6: Merge of *Skor2* and *Nkx6-1* expression. DAPI is used for nuclear staining in all the images. Scale bar: 50 µm

Discussion

DA neurons are a vital source of Dopamine in the Central Nervous System. A defect in these neurons can lead to neurological disorders, including Parkinson's disease. The DA neurons are regulated by GABAergic and glutamatergic neuronal systems. The GABAergic systems inhibits the firing of these neurons, whereas the glutamatergic neurons provide the spark or excitation for the neurons to perform their function. Therefore, damage to systems upstream of the DA neurons could also adversely affect the brain functions. Using single cell mRNA sequencing, the TF *Skor2* co-expressed with *Nkx6-1* and *Vsx2* (Morello et al., 2020), TFs recently shown to mark the brainstem glutamatergic neurons that project to the VTA dopaminergic neurons. This thesis characterizes the expression of *Skor2* in the embryonic brain and mature brainstem nuclei, and analyses its requirement for neuronal differentiation. In addition, this experiment has thrown some light towards *Skor2's* association with glutamatergic neurons.

Skor2 positive neurons label in rv2 domain of early embryonic tissues

Results from the single cell RNA sequencing indicated the presence of *Skor2* positive neurons in the rv2 domain of the early embryonic mouse brain. The same sequencing results suggested a close proximity/affinity towards glutamatergic identity for *Skor2* presence. The results from my experiment showed that *Skor2* is localized in the mantle zone 2 (mz2) area of the rv2 domain. The *Skor2* positive cells co-stained predominantly with *Nkx6-1* in the mz2, while *Vsx2* positive cells near the mantle zone border also express *Skor2*. Previous research from the group (Lahti et al., 2016), showed that the rV2 domain was home to glutamatergic neuronal subtypes marked with *Nkx6-1* and *Vsx2* expression. Thus, consistent with our hypothesis (aim1), *Skor2* is expressed specifically in a subset of rV2 glutamatergic precursors in the developing brain.

Skor2 was not expressed in LDTg at later embryonic stages

The *Nkx6-1* and *Vsx2* TFs are expressed in the LDTg glutamatergic neurons in the later embryonic stages. The early embryonic stage analyses showed that *Skor2* expresses in these glutamatergic neurons, which in conjunction with the earlier statement, suggested that *Skor2* could be expressed in the LDTg. However, my results show that *Skor2* is not expressed in the LDTg. Instead, the *Skor2* positive cells in rhombomere1 are present in a more ventral region, but still co-express *Nkx6-1* and *Vsx2*. Thus, rejecting our hypothesis (aim 1), *Skor2* is not associated with the LDTg.

Skor2 positive cells line up between DTg and VTg in later embryonic stages

While studying the expression of *Skor2*, we observed the presence of *Skor2* in a brainstem region with an unknown function. The area the *Skor2* positive cells occupied has not been molecularly or anatomically well defined. Interestingly *Skor2* was detected in a region between the DTg and VTg, with no overlap with their markers FoxO1 and Fog2 whatsoever. Both the Gudden nuclei VTg and DTg are dense, but project to different mammillary nucleus. This leads to different electrophysiological properties (Dillingham, 2015). The DTg neurons project to the lateral mammillary nucleus and bear head direction cells, which is lacking in the VTg. However, the VTg fires more in rhythm and targets the medial mammillary nucleus. The medial 'theta' system (S.D. Vann, 2009) is understood to play a vital role in memory tasks. The close proximity of the *Skor2* positive cells to the tegmental nuclei suggests there could be a possible regulatory role in controlling functions of spatial awareness and retaining memories.

Skor2 not required for differentiation of their neuronal subtype in the embryonic development

The other aim of the experiment was to study the importance of *Skor2* to cells where it was expressed. To study its importance, we generated homozygous *Skor2* mutants by crossing two heterozygote mutants (Nakatani et al., 2014). Then through immunohistochemistry, I analyzed the knockout with the marker *Nkx6-1* at E13.5 and E18.5 stages. The *Nkx6-1* marker provided very clear results in the heterozygote studies and hence, they were best choice to study the changes forced in the knockout mutant. The results in **Figures 13** and **14** showed no clear distinction between the heterozygote $Skor2^{GFP/+}$ mutant and the homozygote $Skor2^{GFP/GFP}$. The rV2 domain in the early embryonic stage exhibited the same co-expression of the *Skor2* and *Nkx6-1* markers. Similarly, in the later embryonic stage of E18.5, the r1 looked like a facsimile of each other. This leads me to believe that *Skor2* is not vital for the development of these nuclei in the embryonic stage. In previous studies, *Skor2* was understood to be not required for Purkinje cell specification and maintenance of its cell fate (Wang et al., 2011; Nakatani et al., 2014). Thus, our study also portends to a similar belief regarding the cell fate of the *Skor2* nuclei in the brainstem.

Initial design of the study included also studying the changes in comparison with *Vsx2*, but I had troubles achieving consistent signal from the *Vsx2* antibody. Therefore, I focused on the *Nkx6-1* staining, which had previously shown great results and is a very good glutamatergic marker. Another caveat to bear in mind is that due to the time constraint of the project, we were not able study more stages, as was done in the first part of the experiment.

Possible future experiments

The experiment was able to answer certain questions regarding the identity of the *Skor2* positive cells and requirements for *Skor2* TF during neuronal differentiation in the embryonic stages. To push on from what we learned, further studies could be directed towards certain parts in the future. We observed *Skor2* positive cells occupying a certain cluster of cells (later embryonic stages), which to my knowledge have not been studied in detail before. This *Skor2* neuronal cluster has been found to be in proximity to the DTg and the VTg nuclei. The DTg and VTg nuclei play a role in spatial learning & memory retention respectively. Studies regarding the Gudden tegmental nuclei could utilize the *Skor2* cluster knowledge for reference purposes. In addition, due to their proximity, *Skor2* expressing neurons may also regulate the neurons in the VTg and DTg and thus have an impact of spatial learning or memory function.

I could not demonstrate a requirement for *Skor2* during neuronal differentiation. *Skor1* is another member of the *Ski* family as *Skor2.* They are both transcriptional corepressors and both express in rV2 glutamatergic precursors (Morello et al., 2020). Hence, it raises the question of why both? Do they have different roles pertaining to different conditions? The redundancy being a possible point of confusion. To bring clarity, we could study the effect on neuronal differentiation in double mutant knockouts of *Skor1* and *Skor2.*

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